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Alterations in Substrate Utilization in the Reperfused Myocardium: A Direct Analysis by ^{13}C NMR†

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ABSTRACT: An alternative ^{13}C NMR method which allows direct determination of substrate oxidation in tissue for up to three competing ^{13}C-enriched substrates is presented. Oxidation of competing substrates can be measured by ^{13}C NMR spectroscopy under non-steady-state conditions if the relative areas of the glutamate C3 and C4 resonances can be determined. The accuracy of this measurement is limited during brief exposure to ^{13}C-enriched substrates because of the low enrichment in the C3 carbon. The glutamate C4 resonance from a tissue sample which has oxidized a combination of [1,2-^{13}C]acetate (or a uniformly enriched fatty acid mixture) and [3-^{13}C]lactate appears as a nine-line resonance consisting of four multiplet components: a singlet (C4S), two doublets with differing one-bond coupling constants (C4D34 and C4D45), and a quartet (C4Q). It is shown that the sum of the C4S + C4D34 resonance areas versus the C4D45 + C4Q resonance areas directly reports the relative utilization of [3-^{13}C]lactate versus [1,2-^{13}C]acetate, respectively, regardless of citric acid cycle intermediate pool sizes or carbon flux through anaplerotic reactions. We also show that homonuclear ^{13}C decoupling of the glutamate C2 resonance collapses the C3 resonance multiplet into an apparent triplet (actually, a singlet plus a doublet); the relative area of the singlet component reflects the amount of unlabeled acetyl-CoA entering the cycle. The method has been used to determine the contribution of lactate/acetate/glucose to acetyl-CoA in normoxic and reperfused rat hearts. ^{13}C spectra of freeze-clamped heart extracts show quite directly that lactate oxidation is depressed after a 10-min period of global ischemia while acetate becomes the predominant source of energy (60% of the total substrate oxidized through the citric acid cycle). Substrate utilization returns to basal levels (34% acetate, 28% lactate, and 38% unlabeled sources) during 25 min of reperfusion. Ischemic hearts also switch to acetate as the principle source of energy in the presence of a pyruvate/acetate/glucose mixture but not when presented an acetoacetate/acetate/glucose mixture. These results likely reflect control at the level of the pyruvate dehydrogenase complex, which is known to become phosphorylated in heart mitochondria during ischemia.

^{13}C NMR offers considerable potential for monitoring specific biochemical reactions in vivo. The most widely studied pathways so far have been related to glycogen storage and mobilization (Brainard et al., 1989; Jue et al., 1989a,b; Shulman et al., 1990). Since these may be described in most simple terms as a linear biochemical pathways, the exogenously added ^{13}C-enriched substrate (usually [1-^{13}C]glucose) may be followed by NMR as it accumulates in the storage product glycogen or as glycogenolysis is activated to produce end products of the glycolytic pathway which are easily detected, such as [3-^{13}C]lactate. We have chosen to use ^{13}C NMR to examine a variety of biochemical reactions involving the Krebs citric acid cycle (Sherry et al., 1988; Malloy et al., 1988, 1990a). Entry of ^{13}C into a cyclic pathway such as the citric acid cycle inevitably produces intermediates with enriched ^{13}C nuclei in more than a single site within the same molecule (^{13}C isotopomers). This complicates the ^{13}C NMR spectrum as a result of spin–spin coupling between adjacent ^{13}C nuclei, but it also provides valuable biochemical information. We have reported that a complete isotopomer analysis of a single ^{13}C spectrum obtained under metabolic and isotopic steady-state conditions provides information about the relative utilization of competing substrates and ^{13}C flux through various pathways involving the cycle (Malloy et al., 1988, 1990a). More recently, we reported a non-steady-state ^{13}C NMR method for determining relative substrate utilization which could be applied to in vivo situations where the magnetic field homogeneity may not be sufficient to resolve ^{13}C–^{13}C coupling (Malloy et al., 1990b). We now report another simple method which we

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call "the direct C4 analysis" which may be useful for monitoring tissue biochemistry in vivo. We also illustrate the value of homonuclear ^{13}C decoupling to simplify analysis of these spectra. Both methods are demonstrated here by showing how substrate utilization changes with time in perfused hearts following 10 min of global ischemia.

Experimental Procedures

Heart Perfusions. Male Sprague-Dawley rats weighing 300–350 g were anesthetized in an ether atmosphere, and hearts were rapidly excised and placed in 4 °C arrest medium. Hearts were perfused using standard Langendorff methods at a pressure of 70 cm of H_2O by recirculating 125 mL of Krebs–Henseleit buffer. Typical coronary flow rates were 15–18 mL/min. A modified Krebs–Henseleit perfusate containing 119.2 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 10 mM glucose was gassed with 95% O_2/5% CO_2. Hearts were initially perfused with 10 mM glucose as the only exogenous substrate, and ^{13}C-enriched substrates were added to the 125-mL perfusion chamber from a standardized stock solution. Control hearts (n = 4–6 in each group) were perfused with the indicated concentrations of ^{13}C substrates for 30 min before freeze-clamping. Global ischemia was initiated on stable hearts initially perfused with 10 mM unlabeled glucose by stopping all flow to the heart for 10 min while maintaining the temperature at 37 °C by submersing the heart bath chamber in a controlled temperature water bath. The ^{13}C-enriched substrates were added to the perfusion chamber with mixing during the 10-min ischemic period; then flow was reestablished for various periods of time before freeze-clamping (n = 2–3 for each time point). All freeze-clamped hearts were extracted with cold perchloric acid, neutralized with KOH, freeze-dried, and dissolved in 0.5 mL of D_2O for NMR analyses. ^{13}C-enriched substrates were purchased from the following sources: sodium [3-^{13}C]lactate (99%), Isotec; sodium [3-^{13}C]pyruvate (99%), MSD Isotopes; sodium [1,2-^{13}C]acetate (99%), MSD Isotopes; ethyl [2,4-^{13}C]acetoacetate (99%), Icon. The ethyl ester of acetoacetate was hydrolyzed to its sodium salt just prior to use by reacting the ester with NaOH at pH 12 for 2 h at room temperature followed by neutralization.

NMR Methods. Proton-decoupled ^{13}C spectra were obtained at 125.7 MHz on a GN-500 spectrometer equipped with a second broad-band decoupling channel. Heart extracts were scanned in a 5-mm tube using a 45° carbon pulse, 16K data points over 26 000 Hz, and a 6-s delay between pulses and WALTZ decoupling at two power levels (BILEV), and the temperature was maintained at 25 °C using a standard GE variable-temperature accessory. All spectra were zero-filled to 32K points before Fourier transformation. The relative areas of the multiplet components in each glutamate resonance were determined using the GEMCAP curve analysis program supplied with the GE software. Homonuclear ^{13}C decoupling was achieved using single-frequency decoupling generated by the spectrometers F3 channel; the output of this decoupler was connected to the observe coil of the standard 5-mm C/H probe using a lab-built directional coupler.

Results

The glutamate C4 resonance from the ^{13}C NMR spectrum of an extract of a rat heart which had been perfused to isotopic and metabolic steady-state (30 min) with a mixture of [1,2-^{13}C]acetate, [3-^{13}C]lactate, and unlabeled glucose is shown in Figure 1. This single resonance has four multiplet components: a singlet (S) representing those glutamate isotopomers which have an enriched ^{13}C at C4 but not at C3 or

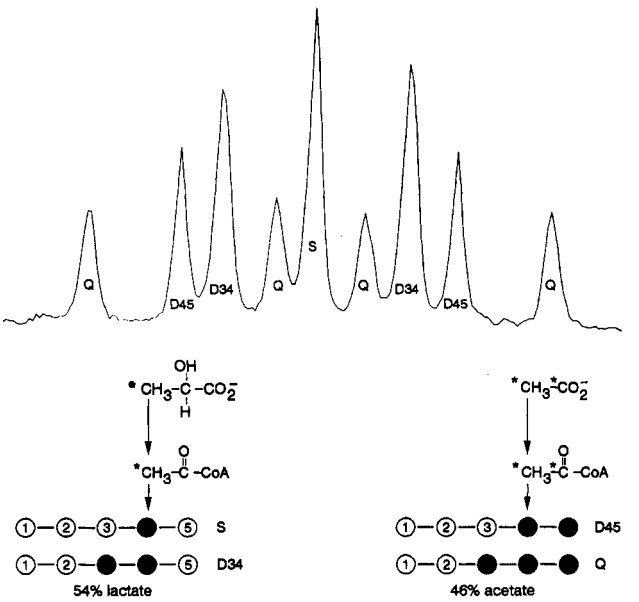

FIGURE 1: Multiplets and isotopomer pools which contribute to the proton-decoupled ^{13}C NMR spectrum of glutamate C4. The areas of the four multiplets (S, D34, D45, and Q) which make up the C4 resonance are proportional to the pools of glutamate isotopomers shown in the bottom panel. This heart was supplied with [3-^{13}C]lactate and [1,2-^{13}C]acetate. Abbreviations and symbols: S, singlet in the C4 spectrum; D34, doublet due to J_{34}; D45, doublet due to J_{45}; Q, quartet or doublet of doublets; filled circles, ^{13}C; numbered circles, ^{12}C.

C5; a doublet (D34) representing those isotopomers with ^{13}C at both C3 and C4 but not at C5; a second doublet (D45) with different J-coupling than D34 representing those isotopomers with ^{13}C at both C4 and C5 but not at C3; and a doublet of doublets or quartet (Q) representing those istopomers with ^{13}C at C3, C4, and C5. The areas of the multiplet components for this heart, expressed as the percent of the total C4 resonance area, are S = 19%, D34 = 35%, D45 = 17%, and Q = 29%.

As illustrated below the glutamate C4 resonance in Figure 1, glutamate C4 and C5 can only be derived from acetyl-CoA as a result of condensation of oxaloacetate with acetyl-CoA in the reaction catalyzed by citrate synthase. Note also that glutamate C1, C2, and C3 are derived from oxaloacetate and the analysis presented above is independent of ^{13}C labeling of the oxaloacetate pool. For example, if every oxaloacetate entering the citrate synthase reaction is unlabeled, then only S (acetyl-CoA from lactate) and D45 (acetyl-CoA from acetate) would appear in the glutamate C4 resonance. If, however, the oxaloacetate pool contained ^{13}C either as a result of citric acid cycle turnover or as a result of influx of labeled molecules into this pool via an anaplerotic reaction, then the D34 and Q components could appear in the glutamate C4 resonance. Again, these later multiplet components are derived from lactate and acetate, respectively. Thus, the sum S + D34 reports the amount of acetyl-CoA derived from [3-^{13}C]lactate, and the sum D45 + Q reports the amount of acetyl-CoA derived from [1,2-^{13}C]acetate, irrespective of the total amount of ^{13}C in the oxaloacetate pool (or any other cycle intermediate) or the size of those pools. Thus, in the example shown in Figure 1, the relative utilization of lactate versus acetate in this heart in quite simply 54% (S + D34) versus 46% (D45 + Q), respectively. The unlabeled glucose or endogenous substrate contribution to the acetyl-CoA pool cannot, of course, by determined by direct analysis of the glutamate C4.

Why then do we find this direct C4 analysis useful, even though it provides only *relative* contributions of two differently labeled competing substrates? First, it does not require a

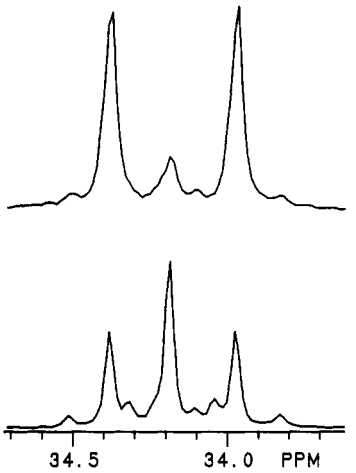

FIGURE 2: Effect of ischemia followed by reperfusion on the proton-decoupled ^{13}C NMR spectrum of glutamate C4. These spectra of perchloric acid extracts were obtained from hearts supplied for 5 min with [3-^{13}C]lactate plus [1,2-^{13}C]acetate (see text). The normoxic heart (bottom) shows a prominent singlet, whereas the dominant resonance in the postischemic heart (top) is the doublet due to J_{45}.

mathematical model; the analysis is simple and unequivocal. Second, the data are obtained from a single ^{13}C resonance and hence applicable to a variety of experimental conditions where comparisons *between* different resonance areas may be problematical; for example, some in vivo spectra may have fatty acid resonances which overlap the glutamate C3 resonance or have differences in T_1 or NOE values that may unknowingly compromise a quantitative comparison of resonance areas. Finally, as illustrated above, this direct analysis does not require isotopic steady-state conditions, and it is independent of anaplerotic flux and changing pool sizes so the measurement may be taken during a wide variety of physiological conditions, being limited only by the time required to collect the NMR spectrum. Since the measurement reflects entry of the various acetyl-CoA isotopomers into the glutamate C4 position *averaged* over the period of time required to collect an NMR spectrum in an intact heart, it would not be sensitive to alterations in flux between acetyl-CoA and glutamate (the first span of the citric acid cycle) which might occur during collection of the NMR data. However, one can only assume that a given mixture of acetyl-CoA isotopomers (derived from various labeled substrates made available to the heart) arrives in the glutamate pool in the same proportions as those which entered the cycle via citrate synthase.

This direct analysis has been used to evaluate changes in substrate utilization in perfused rat hearts during recovery following a 10-min period of global ischemia. The glutamate C4 resonances taken from the ^{13}C spectra of hearts freeze-clamped 5 min after the addition of 1 mM [3-^{13}C]lactate plus 0.25 mM [1,2-^{13}C]acetate to the perfusate of a normoxic heart (bottom) versus a heart recovering from global ischemia (top) are shown in Figure 2. In both spectra, the D34 and Q multiplet components are much smaller than in the spectrum shown in Figure 1 because the isotopomers which contribute to these components are formed somewhat later during isotope turnover through the citric acid cycle intermediate pools. However, inspection of the two spectra shows that the S component clearly contributes much less to the total C4 resonance area in the spectrum of the heart freeze-clamped after the ischemic insult, showing that [3-^{13}C]lactate makes a significantly smaller contribution to the acetyl-CoA pools in heart tissue recovering from ischemia than in normoxic heart tissue. Data for other recovery periods after the 10-min ischemic insult are presented in Figure 3.

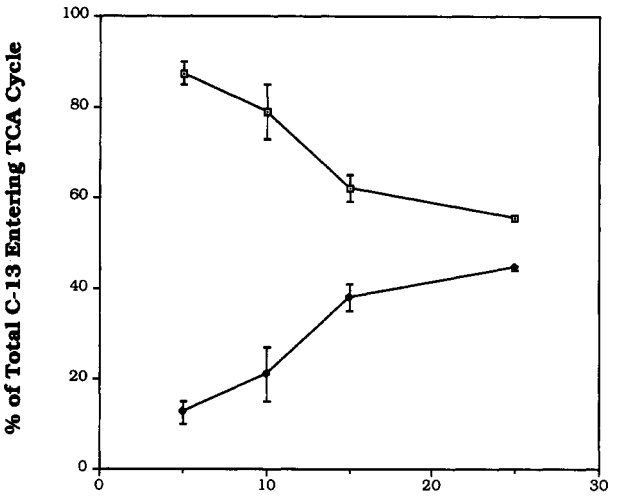

FIGURE 3: Sources of ^{13}C-enriched acetyl-CoA in postischemic hearts. Hearts were supplied with [1,2-^{13}C]acetate and [3-^{13}C]lactate after 10 min of ischemia. Symbols: (□) acetate; (♦) lactate. Each time point represents the average ± SD (n = 2–3).

The data in Figure 3 demonstrate this technique is quite valuable for detecting changes in *relative* substrate utilization between two differently labeled materials, but what about the contribution of unlabeled exogenous substrates or endogenous substrates to acetyl-CoA during recovery? It is possible that the absolute contributions of [3-^{13}C]lactate and [1,2-^{13}C]-acetate to acetyl-CoA both decline following ischemia and they are replaced by unlabeled sources? To answer this question, we need a second measurement which allows an evaluation of the percent of unlabeled carbon sources to acetyl-CoA. One previously presented non-steady-state analysis which allows such an evaluation (Malloy et al., 1990b) depends upon an accurate measurement of D34, Q, and C4/C3 (the ratio of the total C4 and C3 resonance areas). This analysis is quite reliable in most cases but obviously suffers when D34 and Q are low (as is the case in the spectra shown in Figure 2) and when the C4/C3 ratio is large (and changing rapidly with time), as is the case here during the first few minutes after addition of labeled substrates. Another method of determining this same information which may prove useful in such circumstances is illustrated in Figure 4. The glutamate C3 resonance is composed of a singlet (S), two doublets with virtually identical coupling constants (D23 and D34), and a triplet (T). The isotopomer examples illustrated in the figure (bottom) show that unlabeled acetyl-CoA can only contribute to the S and D23 of glutamate C3. If one performs a homonuclear decoupling experiment to spin-decouple the C2 resonance, the D23 component would collapse into a singlet and the T component would collapse into a doublet. The results of this experiment is illustrated in Figure 5 for one heart extract. The area of the central singlet (S′) in this decoupled spectrum reflects the sum of the D23 + S components in the original spectrum and hence provides a direct, quantitative measure of unlabeled versus total labeled substrates which contribute to the acetyl-CoA pool (23% in this example). Given this independent measure of the unlabeled acetyl-CoA pool, the relative acetate/lactate utilization values shown in Figure 3 may be corrected, given the fact that the [3-^{13}C]-lactate + [1,2-^{13}C]acetate + unenriched contributions to acetyl-CoA must equal 1. These data, shown plotted in Figure 6, indicate that in hearts presented with this particular mixture of substrates, acetate is clearly the predominant source of oxidative fuel during the first few minutes after an ischemic

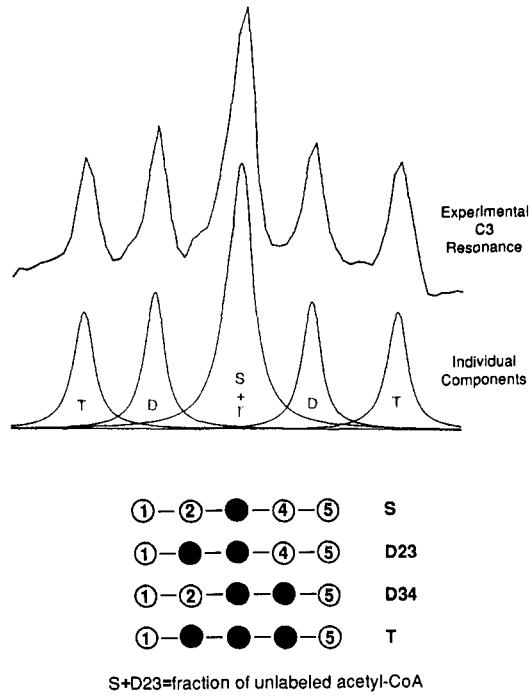

FIGURE 4: Multiplets and isotopomer pools which produce the proton-decoupled ^{13}C NMR spectrum of glutamate C3.

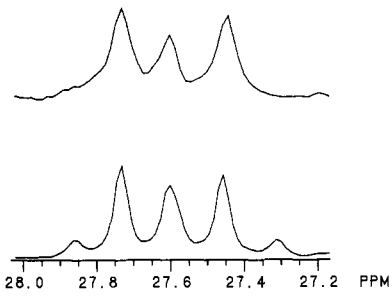

FIGURE 5: Homonuclear ^{13}C decoupling: effect of irradiating glutamate C2. The ^{1}H-decoupled ^{13}C NMR spectrum of glutamate C3 is shown in the lower panel. The spectrum obtained during irradiation of glutamate C2 is shown in the upper panel.

period and lactate oxidation is significantly suppressed. The fraction of unlabeled acetyl-CoA entering the citric acid cycle during this same reperfusion period is nearly constant. Hearts perfused with this same mixture of substrates under normoxic conditions utilized 34% acetate, 28% lactate, and 38% unlabeled sources (n = 4). This indicates that substrate utilization in ischemic hearts returns to basal levels after about 25 min of reperfusion.

Similar data were collected on three other series of ischemic hearts presented with either (a) this same lactate/acetate/glucose mixture plus 5 mM dichloroacetate (sodium salt), (b) a mixture of 1 mM [3-^{13}C]pyruvate, 0.25 mM [1,2-^{13}C]-acetate, and 10 mM unlabeled glucose, or (c) 0.25 mM [2,4-^{13}C]acetoacetate, 0.5 mM [1,2-^{13}C]acetate, and 10 mM unlabeled glucose. Addition of 5 mM dichloroacetate resulted in essentially no change in substrate utilization after ischemia as compared to controls. Hearts provided with the pyruvate/acetate/glucose mixture (data not shown) also showed greater acetate utilization after a 10-min ischemic period, with the quantitative data being virtually indistinguishable from the lactate/acetate/glucose data presented above. Finally, in hearts presented with the acetoacetate/acetate/glucose mixture, utilization of acetate and acetoacetate was virtually constant during recovery from ischemia and not significantly different from utilization rates in the normoxic myocardium

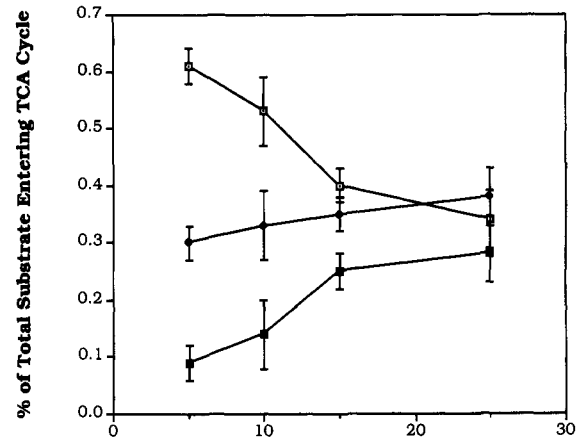

FIGURE 6: Substrate competition in the postischemic heart. The contribution of unlabeled sources (♦), exogenous acetate (□), and lactate (■) to acetyl-CoA. Each time point represents the average ± SD (n = 2–3).

(50% acetate, 44% acetoacetate, and 6% unlabeled sources for this particular concentration of substrates).

Discussion

The non-steady-state ^{13}C NMR method we previously presented (Malloy et al., 1990b) requires measurement of the glutamate C4/C3 ratio and is therefore valid only when the glutamate C3 carbon is sufficiently enriched to accurately measure its area. There are at least two conditions when this measurement might be difficult: either early during citric acid cycle turnover when ^{13}C enrichment of the glutamate C3 pool is low or in certain in vivo situations where there may be other resonances which overlap the C3 resonance. The new, direct non-steady-state method presented here obviates these problems, thereby allowing measurement of relative substrate utilization under a much wider variety of physiological conditions.

The data presented in Figure 3 indicate that lactate oxidation is suppressed while acetate oxidation is enhanced in ischemic rat hearts during the first 25–30 min of reperfusion. This is consistent with inactivation of the pyruvate dehydrogenase complex (PDC) by phosphorylation, which is known to occur in ischemic tissue (Patel & Olson, 1984). Since acetate oxidation bypasses PDC, [1,2-^{13}C]acetate replaces [3-^{13}C]lactate nearly quantitatively as a source of energy immediately after reperfusion. During the first few minutes of reperfusion, PDC is gradually reactivated, thereby allowing reentry of [3-^{13}C]lactate into the citric acid cycle. The results observed with the lactate/acetate/glucose mixture in the presence of 5 mM dichloroacetate and with mixtures of pyruvate/acetate/glucose or acetoacetate/acetate/glucose confirm this interpretation.

Many other investigators have observed similar reactivation of PDC in ischemic heart tissue using classical radiolabeling methods (Dennis et al., 1979; Kobayashi & Neely, 1983; Patel & Olson, 1984). The time required for complete reactivation of the enzyme is quite variable, depending upon perfusion conditions, the substrates available to the heart, cation concentrations, and other factors (Olson, 1989). Kobayashi and Neely (1983) reported that PDC activity returns to control levels after several minutes (data reported for 2 and 5 min only) of reperfusion in the presence of 11 mM glucose and retains full activity during ischemia when 10 mM pyruvate is present. With our experimental conditions (1 mM lactate,

0.25 mM acetate, 10 mM glucose, and 1.25 mM Ca^{2+}), PDC is completely reactivated after about 30 min of reperfusion.

Two other laboratories have recently reported ^{13}C NMR observations in perfused hearts which purportedly reflect flux through PDC. Weiss et al. (1989) have reported that the time for half-maximal enrichment of [2-^{13}C]glutamate is longer in hearts perfused with [1-^{13}C]glucose during low coronary flow (5 mL/min) than during high coronary flow (15 mL/min). This was attributed to lower TCA cycle flux during low flow, a reflection of the known inactivation of PDC under similar conditions (Patel & Olson, 1984). Similarly, Lewandowski and Johnston (1990) monitored the glutamate [2-^{13}C]/[4-^{13}C] resonance ratio in perfused hearts after 10 min of global ischemia and reported that flux of [3-^{13}C]pyruvate into the citric acid cycle is lower in ischemic hearts that in control hearts during the first 3 min of reperfusion. However, these ^{13}C measurements will only accurately report TCA cycle flux (and flux through PDC) if the citric acid cycle intermediate pool sizes (not just the glutamate pool size) are *equal* during control versus ischemic conditions and *constant* during the reperfusion or low flow perfusion periods. There is substantial data in the literature which suggest this may not be the case (Neely et al., 1972; Kobayashi & Neely, 1983). For example, it has been shown that the total citrate concentration can increase 5-fold in hearts perfused with 11 mM glucose between control and 2-min postischemia. This would certainly alter the half-maximal enrichment times in glutamate C2 and the C2/C4 ratio. The direct ^{13}C analysis reported here does not require making assumptions about pool sizes or changes in pool sizes with time; it allows an unequivocal, direct quantitation of relative substrate oxidation in tissue presented with any mixture of singly and doubly ^{13}C-enriched substrates under any metabolic condition over any period of time.

We have also shown that spin-decoupling of glutamate C2 collapses the C3 resonance into a triplet and that the area of the central singlet directly reflects the fraction of unlabeled acetyl-CoA entering the TCA cycle. This technique has been used to show that the amount of unlabeled substrate contributing to the total acetyl-CoA pool in reperfused heart tissue remains relatively constant. This suggests that the source of this unlabeled pool is not exogenously added glucose or endogenous glycogen, since inactivation of PDC would also prevent glucose or glycogen from contributing to the acetyl-CoA pool. Although it remains to be seen whether this ^{13}C homonuclear decoupling technique will offer any practical advantages over measuring the C4/C3 ratio during in vivo ^{13}C observations, it does provide an alternative approach which may prove useful in some circumstances.

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